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Factors controlling hop flowering and their potential and their potential for use in the brewing and pharmaceutical industries

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FACTORS CONTROLLING HOP FLOWERING AND THEIR POTENTIAL FOR USE IN THE BREWING AND PHARMACEUTICAL INDUSTRIES

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Introduction

Hop (*Humulus lupulus* L.) is a dioecious, perennial vine grown in temperate regions across the world (Stevens et al., 1997). Today hop is being used for two purposes. The first and most prolific is its use in beer brewing. The other is a recent development, but ever growing interest in the use of hops for pharmaceuticals.

In beer brewing female hop cones (hops) are used as a natural preservative and as a source of bitter flavors (Milligan et al., 1999). During the brewing process hops are first added to the wort early on to contribute to the colloidal (uniformity) and bacterial (preservation against bacterial growth) stability of the beer. More hops may be added at the end of boiling, and/or just before packaging in order to add a signature bitter or "hoppy" flavor and aroma. Terpenoid compounds including alpha-acids, also known as humulones, serve as the source of these flavors and aromas while beta-acids, or lupulones yield the sterility preserving biosteric effects (Keukeliere, 2000). Different varieties of hops can contrast in both the proportion and constitution of the various acidic compounds and their variable side-chains making for a multitude of different flavors and aromas. Even within a single hop variety differences in growing conditions can also affect the resulting flavor (Keukeleire, 2000). In the past, hops varieties were created based on the location and condition of the area they were grown.

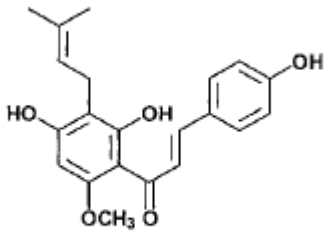
Today more and more emphasis is being placed into understanding exactly what compounds create the desired flavors. For instance alpha-acids, namely humulone and cohumulone, are converted to iso forms during wort boiling which impart the desired acids for bitter flavors in beer. Also it is noted that isocohumulones tend to impart a harsher bitter flavor

than other isohumulones (Keukeleire, 2000). Therefore if a brewer wants to impart a specific flavor into their beer it would be beneficial to be able to engineer flowers to produce fewer β-acids and more α-acids to reduce the amount of hops needed to impart flavor. Also the flowers could be made to produce more or perhaps fewer cohumulones thus changing the 'harshness' of the bitter flavor.

Another current emphasis in hop research is how to enhance the production of commercially valuable compounds within hop cones through genetic engineering. This type of experiment has already been conducted in Washington (Hopsteiner Inc., pers. Commun.) where two types of hops, Willamette and Zeus, were sprayed with 27.5% and 10% solutions of the compound calcium 3-hydroxy-5-oxo-4-propionyl-cyclohex-3-encarboxylate (Pro-Ca) trademark named Apogee and Regalis, respectively. Pro-Ca is known for its ability to inhibit enzymes necessary for the production of gibberellins, ethylene, and several classes of flavonoids (Kavalier et al., Lehman College, 2010 [unpublished]). In a three week study, Willamette plants treated with 50 ppm of Pro-Ca during the first week increased flower yield by 14.6% compared to controls (Kavalier et al., Lehman College, 2010 [unpublished]). Plants treated in the second or third week also showed increases, but results were not statistically significant. However, Zeus treated with Pro-Ca did not show any significant change in yield due to complications with natural variation among clonal plants (Kavalier et al., Lehman College, 2010 [unpublished]). It is also possible that the effects of Pro-Ca treatment are highly variable and cultivar-specific, as is seen in apples (Rademacher et al., 2006). In another test, high throughput ultra high performance liquid chromatography (HTS UHPLC) was used to examine the effects of 50 and 100 ppm dosages of Pro-Ca on hops phytochemical constituents, namely terpenophenolic

compounds. The test identified several compounds including the prenylflavanoids xanthohumol, and desmythylxanthohumol, α-acids cohumulone, humulone, and adhumulone, and β-acids colupulone and lupulone. Zeus treated with Pro-Ca shows a 39.1-48.6% increase in the prenylflavanoids and an increase of 30.5-46.7% in α- and β-acids (Kavalier et al., Lehman College, 2010 [unpublished]). Treatment of cultivar Willamette did not reveal any significant results again showing that the effect of Pro-Ca is cultivar specific. Overall while the effect for specific cultivar must be individually tested. Pro-Ca shows the ability to increase yield and boost the production of brewing desirable α-acids and pharmaceutically significant compounds such as xanthohumol.

Further testing to discover additional effects and the cultivar specific optimal dosage of Pro-Ca for specific treatments will be required before these chemicals can be put to commercial use. As of now those tests could take years to complete as hop only flowers once a year. By understanding the mechanisms of hop flowering, it may be possible to devise a protocol for forced flowering *in vitro* that would allow preliminary tests to be done throughout the year on a small scale. Once optimal doses are determined during the non-flowering months via forced flowered plants, large field studies can be done during the next flowering season to confirm or deny results. The ability to run several tests in a single year could drastically reduce the time needed to put Apogee or other modifiers like it on the market.



Xanthohumol (XN)

Figure 1: Structure of xanthohumol

Source: Modified from Gërhauser et al, 2002

The most recent point of interest for the use of hops has come about due to the pharmaceutical potential of some secondary compounds. Compounds showing the most promise are xanthohumol (Figure 1) and 8-prenylnaringenin, which are prenylflavenoids produced in the female hop cones (Schaefer et al., 2003). 8-prenylnaringenin shows promise as a potent phytoestrogen compound capable of treating human post-menopausal symptoms such as hot flashes and osteoporosis (Stevens & Page, 2004). Xanthohumol is especially interesting because it has been found to cause inhibition at the initiation, promotion, and progression stages of carcinogenesis (Gerhauser et al., 2002). It is a broad-spectrum chemopreventative agent, and shows potential as a cancer combative medication through a variety of effects. The effects associated with protection against initiation of cancer are its ability to modulate the activity of enzymes involved in carcinogen metabolism and detoxification, and the capability of scavenging reactive oxygen species (electron deficient and incredibly reactive oxygen molecules capable of damaging human tissue and DNA). The promotion protection effects of xanthohumol include anti-tumor promoting mechanisms, and anti-inflammatory properties through the inhibition of a variety of enzymes. Finally xanthohumol also shows the

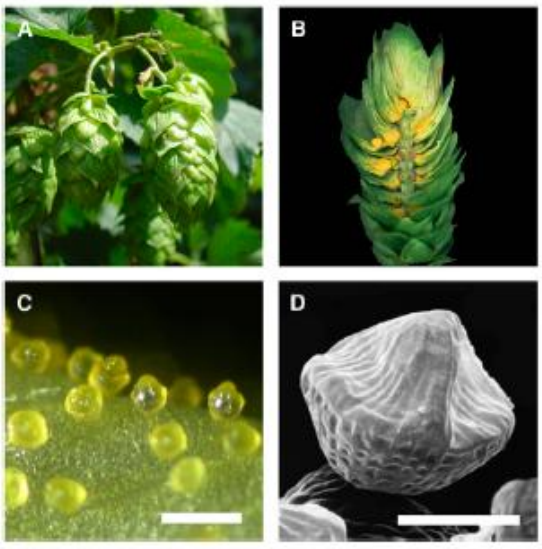


Figure 2: Morphology of Hop Cones and Lupulin Glands.

(A) Cones of hop cultivar Taurus. Cones are ~5cm in length.
(B) Longitudinal section of a hop cone showing lupulin glands at the base of bracteoles.
(C) A light microscopy image of ripe lupulin glands. Bar = 100µm.
(D) Scanning electron micrograph of a ripe lupulin gland showing the peaked appearance of the filled subcuticular sac. Bar = 100 µm.

Source: Nagel et al, 2002 *et al* 2008

ability to protect from cancer proliferation by inhibiting DNA synthesis while inducing cell cycle arrest at the S phase, apoptosis, and cell differentiation (Gerhauser et al., 2002).

In hops, xanthohumol is produced in lupulin glands of female flowers (Figure 2). These glands are secretory tricomes generally located at the base of flower bracteoles (Stevens & Page, 2004). The issue is that the amount of xanthohumol naturally produced in each flower is about 0.95% of its dry weight. This minuscule amount is very difficult to extract and overall the cost of harvest is not effective for the volume needed for pharmaceutical use (Nagel et al., 2008).

Presently there are four main procedures for attempting to increase the amount of xanthohumol in plants. The methods are selective breeding, chemical synthesis, microbial production, and genetic engineering. The most commonly used method for trait selection is selective breeding, and this method has been effective in increasing the production of other hop metabolites (Neve, 1991). However, it has only been effective for increasing xanthohumol production from 0.95% to 2% which makes it possible to extract, but still too inefficient for pharmaceutical use (Renault et al., 2006). Many pharmaceuticals that were once extracted from plants are later created synthetically to reduce cost. While it is possible to chemically synthesize xanthohumol from phloracetophenone, the yield is only about 10% (Khupse & Erhardt, 2007) which is neither efficient nor high enough to be a cost-effective procedure. It is also possible to use microbial agents such as bacteria or fungi to assist in the production of chemicals outside the plant itself. This was attempted with hops by introducing the gene responsible for making O-methyltransferase, the enzyme that synthesizes xanthohumol, into *E.*

coli with the hopes of creating a xanthohumol producing biomachine (Nagel et al., 2008). However, the protein was insoluble and non-functional in *E. coli* and also led to cell death (Gerhauser et al., 2002). If xanthohumol was ever successfully produced using this method, new *E. coli* microbes would be required for each batch.

Of the methods proposed to attempt to amplify the amount of xanthohumol produced by hop cones, the most promising to date seems to be that of genetic engineering. In 2010 research was conducted in an attempt to try and introduce the structural gene Cinnamate 4-hydroxylase (C4H) via *Agrobacterium temufaciens*-mediated transformation in order to amplify the amount of xanthohumol present in female hop cones (Kettleson, 2010). The gene was introduced in conjunction with GFP to serve as a marker of successful transformation (Kettleson, 2010). While no transgenic plants were indentified, the process is very promising as a method to increase ease and efficiency of harvesting xanthohumol for pharmaceutical research.

The major setback of research pertaining to the genetically engineered transgenic production of xanthohumol is determining whether or not the transgene, once successfully introduced into the plant, will achieve the desired effect in the cones. Presently the process for testing hops for successful transformation is incredibly lengthy (Schwekendiek et al., 2007). *In vitro* callus cultures of hops that have had the transgene introduced must be first be grown into shoots (usually 3-5 cm in height) that have begun rooting. They can then be transferred into sterile soil beakers to grow further. After 1-2 months the soil plants can be moved into unsterile greenhouse conditions where a labor intensive watering regime must be maintained to keep

the newly introduced plants from drying out from increased exposure to air. Once acclimated, the hops must be allowed to grow naturally for at least two growing seasons before flowering can be achieved. Overall the process from production of transgenic callus to flowering is at minimum a two year venture.

Because the major production site for xanthohumol and other pharmaceutically active compounds are the female cones, this flowering process must happen in order to investigate the effect of the newly introduced transgenic traits. This complex process forces the research into xanthohumol production to take considerable time. If a process of forced flowering, or the induction of flowering on demand, was successfully created, its implementation could yield multiple benefits. For one this research, if successful, could allow for important advancement in cancer research. Also as most of the research is presently being done by undergraduate and graduate students at the University of Northern Iowa a two year turnover rate means multiple generations of students would be required to conduct a single experiment. Overall, through the understanding of hop flowering and the development of an *in vitro* forced flowering procedure a reduction in the time it takes for hop to flower could greatly speed the transgenic research process. Subsequently, this could increase the chances of developing upregulated xanthohumol producing hops that could be used to further the development of anti-cancer pharmaceuticals. The first step in the development of a forced flowering procedure to be used for the benefit of both the brewing and pharmaceutical industries is to understand the natural mechanisms of hop flowering.

Hop flowering is induced by a complex interplay of environmental and biological cues. The three main players in the flowering process stem from the regulation of light, temperature, and hormones (Bernier et al., 1993). By understanding the natural process of hop flowering and the different factors that control it, it may be possible to devise new ways to control the flowering process, perhaps to the point of inducing flower development on demand; or forced flowering. If such a method was devised it could prove to be invaluable to the brewing community and pharmaceutical research by allowing for the fast development of α-acid rich high yield hops for brewing and xanthohumol overproducing hops to be used in pharmaceutical research.

Based on the potential benefits controlled flowering could have for the brewing and pharmaceutical industries, the goal of this research was to review the main factors controlling hop flowering. The purpose of this research is to be able to devise potential strategies to help future researchers successfully direct induce flowering in hop. The three main factors focused on were the effect light, temperature, and hormones have on flowering regulation.

Literature Review

Light Regulation

It is a common fact that light is required for plants to live and grow; it is an integral part of photosynthesis among other biological functions. It is less well known that light also plays an imperative role in plant flowering. Plants can be divided into three categories based on how light is used in the flowering process. These categories are long-day, short-day, and day-neutral. The “day” portion is referring to how much daylight, or sunlight, is received by the plant

compared to the amount of darkness (Kobayashi & Weigel, 2007). This is often expressed in an hour-interval with the hours of daylight proceeding the hours of darkness.

Long-day plants are so named because they will only flower when they receive enough light to equal or exceed a specific minimum threshold (Kobayashi & Weigel, 2007). In other words, once the days become long enough, flowering can begin, any shorter and flowering is repressed. Short-day plants are the exact opposite. These plants will only flower once the amount of light received has fallen below a maximum threshold. Or, once the days become short enough, they can flower. Any longer and flowering is repressed (Kobayashi & Weigel, 2007). However, these rules are not absolute, long-day plants in continuous light and short-day plants with too little light (which is often species or even genotype specific) will also not flower, even though the amount of light is above or below their respective thresholds. It is also important to again keep in mind that since short-day means light must be below a critical threshold and long-day means it must be above, it is possible for the critical daylength of a short-day plant to be quite a bit longer than that of a certain long-day one (Thomas & Schwabe, 1969; Kobayashi & Weigel, 2007). Lastly, it is nearly always the case that several days of the proper light interval must be experienced before the flowering process can be induced. Flowering is best if the light interval is maintained up and through flowering; however, with some plants it is possible to induce the plant in a few days before moving it to a different light pattern and flowering will still occur.

The final category is day neutral plants. These plants generally flower best under plant-specific optimum light conditions, but will eventually flower regardless of the pattern of light

around them (Kobayashi & Weigel, 2007). This seems to show that light does play some role in the regulation of flowering, but improper light will not entirely shut the process down. However, just knowing specific amounts of light are required for plants to flower does not tell us how these plants are capable of telling the difference between night and day and the interval between the two.

A plant's ability to determine when to flower depends on two processes; its ability to detect and react to light and its ability to relate light to a 24 hour cycle. Plants have many receptors on their cell surface for a variety of functions. Two such photoreceptor proteins, phytochrome and cryptochrome are used by plants to detect light (Hoang et al., 2008; Hughes & Lamparter, 1999). It has been postulated that the light received by these proteins is used as energy to promote the production of flowering repressing hormones during the day. Then during the night these hormones degrade (Kobayashi & Weigel, 2007). The hormones themselves will be discussed later.

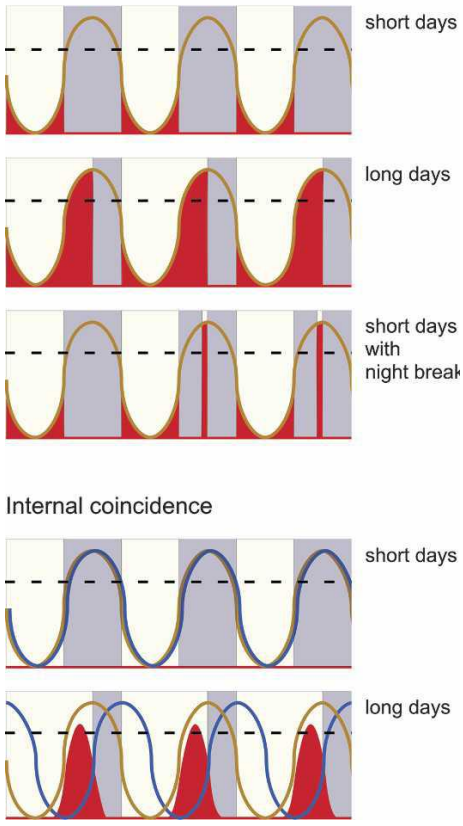


Figure 3: External and Internal Models for Photoperiodism

Two models to explain photoperiodic responses in short-day plants. In the external coincidence model, light entrains a periodic function (ochre). Light also causes the production of a repressor (red) directly proportionate to this function. Thus, even short night breaks, if given at the correct time, can lead to the repressor surpassing a critical threshold (dashed line). In the internal coincidence model, light signals (e.g., lights on/off) entrain two different periodic functions. If one function (ochre) leads to production of a repressor, but the other function (blue) inhibits it, it will depend on their relative overlap whether the repressor (red) will reach a critical threshold. Since the details can vary considerably, it is difficult to predict the effects of night breaks on an internal coincidence mechanism.

Source: Kobayashi & Weigel, 2007

We have seen with plants such as sunflowers that they are capable of following the sun throughout the day. It has even been seen that juvenile bean plants will bend their leaves in a specific pattern during the day, even when kept in complete darkness (Holdsworth, 1959). While it has not been fully confirmed, the best supported reasoning for this phenomenon is that plants have some sort of circadian or biological clock. Generally this clock is 24 hours, though a few other lengths have been recorded in some strains of beans. There are two explanations, the external and internal models that can explain how the interaction of an internal clock and external light leads to either flowering or flowering repression (Figure 3).

In the external model the clock is divided into two phases, the photophile or light-sensitive phase, and the scotophile or dark-sensitive phase (Kobayashi & Weigel, 2007). A switch between these two phases creates an oscillating pattern. This internal clock could then determine the length of day by the presence or absence of light during each phase. The photophile phase of a short-day plant would be approximately as long as its optimum flowering daylength. Daylengths that are too long would result in light present during the scotophile phase (Kobayashi & Weigel, 2007). A long enough duration of light would lead to the production of flowering repressing hormones past a critical point and flowering would be repressed (Kobayashi & Weigel, 2007). In a long day plant daylengths that are too short would result in darkness during its photophile period. Again, if this mismatch in external light and internal phase exceeded a maximum threshold, flower production would be repressed (Kobayashi & Weigel, 2007). Also night breaks, or the addition of short burst of light during the night can be enough to repress flowering (Kobayashi & Weigel, 2007; Bunning & Stern, 1930; Pittendrigh, 1960).

The internal model considered the presence and absence of light as each creating its own oscillation pattern. One of these would result in flowering repressor production, the other in flowering repressor inhibition. The amount of overlap between the two (as the points that overlap would cancel) that would determine whether or not flowering would be repressed (Figure 3). Overall, only when those two patterns are in-sync would the plant be able to flower (Kobayashi & Weigel, 2007; Pittendrigh, 1972).

Hop (*Humulus lupulus*) flowers as daylengths begin to shorten in midsummer and are generally referenced as having a 10 hour photoperiod which is assumed to be 10 hours of light and 14 hours of darkness (Pharis & King, 1985). The optimum light pattern for hops flowering is debatable as the exact daylength is variable and generally genotype specific.

The effect of light on flowering and the optimal patterns to produce the fastest flowering was examined by Thomas and Schwabe (1969). Experiments tested three genotypes of hops; Fuggle, CC 31, and New York Hop. One experiment led to the conclusion that hop is a short-day plant. This was based on the definition that short-day plants only flower once daylengths become sufficiently short, with daylight hours being less than some maximum threshold. They also found that hop will become dormant under very short daylengths. After correcting for the partial light periods during dawn and twilight Thomas and Schwabe (1969) tested 8, 13.5, 16.5, 20.5, and 24 hour photoperiods with the 24 meaning continuous light. A 28 week experiment showed that the 13.5 hr and 16.5 hr flowered in 12 and 26 weeks respectively while the 20.5 hr and 24 hr was too long and repressed flowering although they continued to grow vegetatively. The 8hr stopped growing all together after the 8th week and became

dormant. This showed that there is both a minimum and maximum number of hours allowable for hops to flower. The overall range of photoperiods in which hops will flower seems to be between 10 and 16 hours of daylight. With genotype Fuggle it was shown that while the longer photoperiods of both 14 and 16 hours succeeded in producing inflorescences, the 14hr photoperiod flowered terminally while the 16hr flowered in both the terminal and auxiliary positions although it was slightly delayed. These observations lead to the conclusion that there may be a tradeoff between number of flowers and time taken to flower and also that each genotype could have a photoperiod wherein it flowers fastest.

Night breaks as short as 30 minutes are effective in repressing flowering of hop under short-day conditions (Thomas & Schwabe, 1969). These breaks have the effect of artificially lengthening the day and push the hop in question past their critical daylength. However, if the night break serves to only slightly pass the critical the plants may flower after the night breaks have ended, even if they are no longer under short-day conditions. Even though they may flower, the few plants that still do so can be delayed upwards of a month and a half and may produce some abnormal flowers (Thomas & Schwabe, 1969).

It is important to note, that not any plant can be induced into flowering via short-day photoperiods. Hops must achieve a genotype specific minimum node number before they can be induced (Thomas & Schwabe, 1969). For the genotypes Fuggle, CC 31, and New York Hope these numbers are approximately 23, 12, and 20 visible nodes respectively. Once the minimum node number is reached, as little as one week of short-days is sufficient to induced flowering,

although the bloom is often delayed compared to 2 or 3 weeks of short-days. Also less than one week of short-days is ineffective for inducing flowering in hops.

Temperature Regulation

Many plants use cues from the environment to determine the proper season for flowering (Sung & Amasino, 2006). As plants are naturally grown outdoors the temperature range in which they flower is simply the average temperature range of their geographic location during flowering season. Therefore changing temperatures will have more effect on the overall health of the plant than it will in promoting or repressing flowering alone. This is not to say that flowering and daily temperature are totally independent of each other. It has been seen that lower temperatures can allow plants to flower in abnormal daylengths. For instance in an experiment where the hop were grown outdoors versus inside a greenhouse; hop has been shown to flower in daylengths longer than the critical daylength if they were kept in a cooler setting (Thomas & Schwabe, 1969).

Where temperature really comes into play with plants such as hops is in the transition from juvenile to mature plants. Many of these plants, hops included, undergo an interesting phenomenon called vernalization. Vernalization is a process in which plants endure a prolonged period of cold temperatures, usually winter, which cause changes in the plant that jumpstart a plants ability to flower come springtime (Sung & Amasino, 2006; Müller & Goodrich, 2001). Without the cold period, or cold shock, even with proper springtime light conditions the plants will be unable to flower.

The cold-sensing mechanism has not been studied in depth, but it is known that this mechanism sends cellular signals that trigger the expression of dormant genes. In *Arabidopsis* one such gene essential in the vernalization process is VERNALIZATION INSENSITIVE 3 (VIN3) (Sung & Amasino, 2006). This gene is only expressed in the presence of cold and the amount of expression is correlated to both the duration of the cold period and how strong the vernalization response is. It is likely that hops also use either VIN3 or a species specific homologue in its own vernalization process.

The changes that occur during and after vernalization are not fully understood, but seem to have both genetic and epigenetic components. In many plants that require vernalization the major repressor of flowering is a gene by the name of FLOWERING LOCUS C (FLC); (Sung & Amasino, 2006, Mouradov et al., 2002). FLC works by repressing the flowering promoting genes FLOWERING LOCUS T (FT) and SUPRESSOR OF CONSTANS1 (SOC1); (Müller & Goodrich, 2001). The vernalization process reduces the levels of FLC via a Polycomb Group gene (PcG) by the name of Polycomb Repression Complex 2 (PRC2) and allows FT and SOC1 to be expressed (Sung & Amasino, 2006).

One model for how vernalization leads to these changes in gene expression is the histone code hypothesis (Sung & Amasino, 2006). In this model the covalent modification of histones leads to changes in chromatin states that result in an increase or decrease in levels of gene expression. For example a presently expressed gene may coil into a highly condensed heterochromatin unfavorable for expression while another may convert to a more open and relaxed euchromatin state leading to an increase in expression (Sung & Amasino, 2006). This

model is supported by the fact that the level of PcG-mediated methylation at histones H3K9 and H3K27 both increase at FLC chromatin during vernalization (Sung & Amasino, 2006). This would suggest FLC is being wound more tightly leading to the decrease in expression noted above.

PcG components seem to lack specific DNA binding activity, yet they are actively recruited to act on specific gene targets such as FLC (Müller & Goodrich, 2001). Recently a mechanism for this process involving noncoding RNA (ncRNA) has been introduced (Müller & Goodrich, 2001). Two noncoding RNAs termed COLDAIR and COOLAIR have been found to originate from within the FLC sequence (Müller & Goodrich, 2001). One PcG complex that includes PRC2 actively binds the COLDAIR ncRNA bringing it in close vicinity to FLC. COOLAIR is upregulated by the presence of cold temperatures and seems to work by reducing transcription

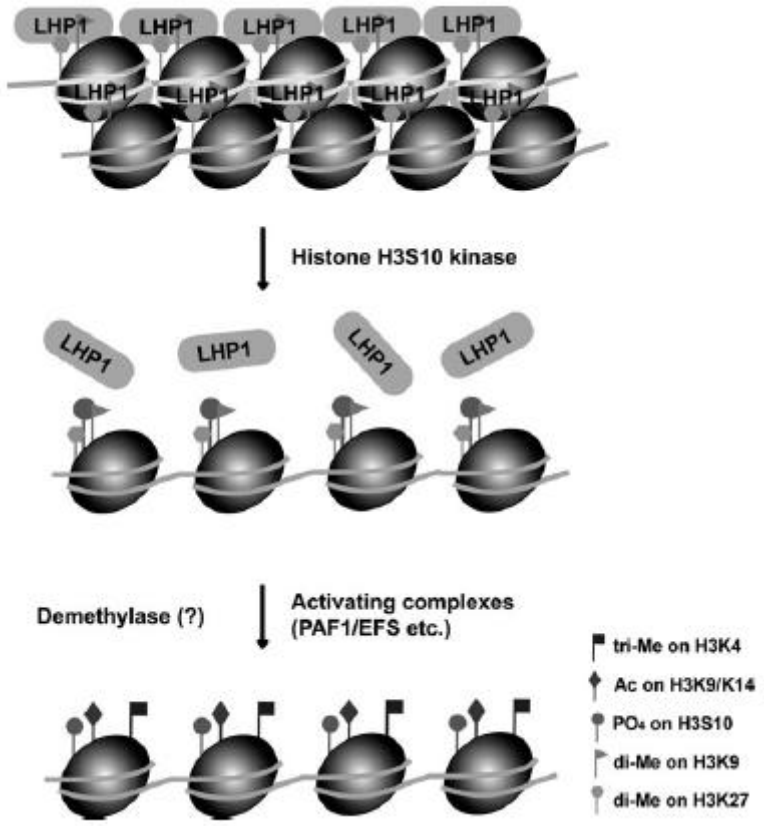


Figure 4: Hypothetical mechanism for resetting FLC chromatin during meiosis.

Vernalization causes LHP1 to associated with FLC chromatin and to 'lock-in' stable FLC repression as discussed in the text (top). Increasing phosphorylation at H3S10 could prevent LHP1 from binding (middle). Activating components gain access to FLC chromatin to reactivate FLC expression in the next generation (lower).

Source: Sung & Amasino, 2006

of the FLC promoter but its mechanism and exact function in vernalization is still unclear (Müller & Goodrich, 2001).

For most species vernalization is permanent throughout a plants life cycle; however, in the next generation of hop FLC becomes reactivated, returning the new plants to their juvenile state and the new plants are unable to flower until they also become vernalized. Again no mechanism has been confirmed, but the most likely candidate is a reshuffling of repressive proteins during the flowering process. As flowering occurs there are numerous rounds of cellular division thus requiring regions of heterochromatin to relax so DNA replication can take place (Sung & Amasino, 2006). Therefore it is likely that various kinases could gain access to various heterochromatins including the repressed FLC region and phosphorylate, returning them to the original relaxed state (Figure 4).

Hormone Regulation

The ultimate effect of both light and temperature is to trigger the production of chemical compounds that directly facilitate the growth of floral structures. It is widely believed that these chemicals used by plants are hormones. Plant hormones or phytohormones are defined as growth regulators and play some part in the regulation of nearly every portion of plant development (Hirsch et al., 1997). An old but still argued idea for how hormones regulate flowering is the florigen hypothesis (Chailakhyan, 1979). Florigen is believed to be a complex of many different hormones that work in a bicomponent nature. The first half of the bicomponent complex is made up of hormones called gibberellins which stimulate the growth of flower stems. The second half is describe as anthesins, which are chemicals believed to cause the

formation of flowers (Chailakhyan, 1979). Whether or not the florigen hypothesis is correct, there are three main classes of hormones that have been shown to play a large part in plant growth and development, including the process of flowering. These three hormones are gibberellins, auxins, and cytokinins.

Cytokinins generally resemble the nucleic acid adenine in form and are responsible for the stimulation of cell division and morphogenesis, or the development of shoots and/or buds (Werner et al., 2001). The most common naturally occurring plant cytokinin is zeatin (Figure 5).

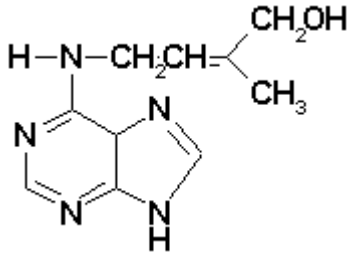


Figure 5: Chemical structure of Zeatin

Source: http://www.plant-hormones.info/cytokinins.htm

Auxins come in a variety of forms, and all of their functions center around growth. They mainly influence aspects of cell division, cell elongation, and cell differentiation (Teale et al., 2006), but the exact mechanism is yet to be understood. The three most common auxins found in nature and/or utilized by humans are indole-3 acetic acid also known as IAA (Figure 6), 1-napthaleneacetic acid (NAA), and 2,4 – dichlorophenoxyacetic acid (2,4-D). Of the three, IAA is the most important naturally occurring auxin and is predominant in plants (Teale et al., 2006). IAA was first isolated and identified as a messenger between the initial perception of light and the physical bending of a

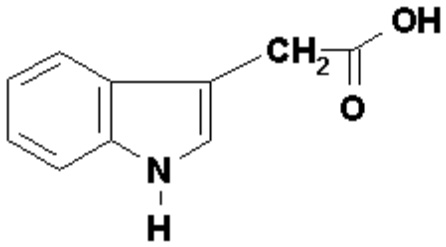


Figure 6: Chemical structure of indole-3-acetic acid (IAA)

Source: http://www.plant-hormones.info/auxins.htm

plant towards the sun. This seems to indicate that IAA is produced in the presence of light and then migrates elsewhere to mediate cellular biological functions.

Gibberellins are naturally occurring acids found in plants (Hooley, 1994). They are categorized both on the basis of structure and function with the main structure being a gibberellin skeleton (Figure 7). All gibberellins are named Gibberellic Acid (GA) and are distinguished from one another by numbered subscripts ordered by time of discovery. Gibberellins have many widely known functions including internode elongation, flower induction, and modulation of sexual expression (Villacorta et al., 2008), with their main purpose generally accepted as the regulation of vegetative growth. This is supported by past experiments which show that mutant dwarf plants treated with exogenously supplied gibberellins will grow to the normal height of non-dwarf varieties (Chailkhyan, 1979). In fact, the dwarfness of the plants was caused by a lack of production of their own gibberellins causing unevenness between growth promoting gibberellins and other growth inhibitors.

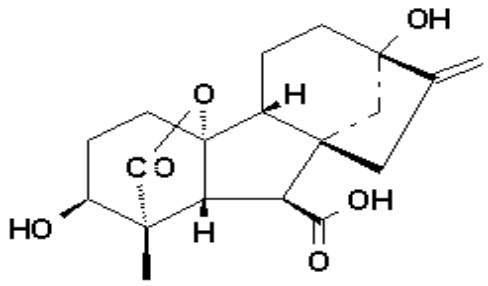


Figure 7: Gibberellin skeleton (GA_1)

Source: http://www.plant-hormones.info/gibberellins.htm

Research in *Arabidopsis* mutants has shown the gibberellins GA_1, GA_4, and GA_5 to primarily affect flowering (Guan et al., 2006). It is thought that gibberellins are responsible for the activation of the LEAFY gene which determines flower meristem identity. This flowering effect has been shown in other species such as *Lolium temulentum* in which a many-fold increase of both GA_1 and GA_4 were seen at the shoot apex during inflorescence development

(Guan et al, 2006). More importantly this same effect was also seen in hops (Villacorta et al., 2008).

During initial growth, young hops show gradually increasing levels of GA_1 until the time of flowering initiation at which point it sharply decreases and shows the lowest levels during flower development (Villacorta et al., 2008). GA_3 and GA_4 show dramatic increases in levels in plants 4-5 m high (Villacorta et al., 2008). This is important because that height is the maximum for vegetative development before macroscopic inflorescences become visible (Villacorta et al., 2008; Revilla et al., 2007), or in simple terms the levels of GA_3, and GA_4 especially, skyrocket just as hops transition into the flowering stage. However, the relationship between gibberellins and flowering seems to have less to do with their individual levels and more to do with the ratio between gibberellins, auxins and cytokinins (Revilla et al., 2007). Accompanying these gibberellin patterns, the cytokinins t-zeatin (t-Z) and iso-penteniladenine (iP) increased gradually during vegetative development, maxing out in the transition from vegetative to reproductive growth, and then dropping off during floral development. IAA is present in the highest amounts of all hormones and follows a profile similar to GA_1 increasing and then dropping off as floral development begins (Revilla et al., 2007). This type of balancing is also seen in grapes where higher gibberellin levels initiate the formation of the inflorescence axis towards the peak of vegetative growth, and then transition to higher cytokinin levels triggering the differentiation into flowers (Srinivasan & Mullins, 1980).

In exogenous application, GA_3 is effective at mimicking the effect of cold temperatures although the minimum effective levels have not been determined (Pharis & King, 1985). Also

the application of GA_3 to green organogenic nodular clusters (GONCs) results in increased shoot inductions as well as a doubling in callus growth (Schwekendiek et al., 2009). The shoots of callus exposed to GA_3 for a period of two weeks of greater degenerate into a not fully identified distorted flower precursor (Schwekendiek et al., 2009).

Much of the testing to attempt to induce flowering must be done in a lab. The best *in vitro* tissue to work with is callus. Although it seems to have no effect on flowering induction, thiodiazuron (TDZ) is essential for the growth of green organogenic calli to be used in experiments. 4.54 µM TDZ is sufficient for inducing green callus growth from intermodal stem pieces without the aid of any other chemical (Schwekendiek et al., 2009).

Summary

While the exact mechanism for how light regulates hop flowering is yet to be documented, it is generally accepted that hops plants have an internal circadian clock of sorts that allows them to determine external daylength. Hop flowers as daylenghts move from longer to shorter usually in mid to late summer. The Florigen, External Coincidence, and Internal Coincidence hypotheses all attempt to explain why this is, and while none have been fully confirmed, it seems that flowering is kept in check by a balance of flowering repressors and promoters that react to light. Once the period of daylight reaches the proper length, the amount of light reduces levels of flowering repressors to drop below the levels of promoters, thus allowing flowering to take place. The proper daylength for hops flowering is genotype specific but ranges from approximately 10-16 hours of daylight, although slightly longer days can still induce flowering if temperatures are abnormally high.

Temperature at the time of flowering does not seem to play a major role in controlling the flowering of hops. However, hops are required to go through an extended period of cold temperatures, or vernalization, before flowering can occur. In *Arabidopsis* two non-coding RNA sequences COOLAIR and COLDAIR are upregulated in the presence of cold temperatures, likely through methylation and relaxation of local chromosomes. These sequences actively block the flowering repressor gene FLC thus allowing for hops to flower. However, the vernalized state is not maintained across generations due to a natural reshuffling of repressive proteins during the prolific cell division during flowering. It is possible that a similar mechanism also controls hop vernalization; however, there is presently no research to confirm or deny this possibility.

Hormone levels have the most direct control over hops flowering. The ability for hops to flower stems from a balance between gibberellins, auxins, and to a smaller extent cytokinins. It is likely that the flower repressors and promoters hypothesized to be produced during light cycles are actually these hormones. During natural flowering t-Z, iP, IAA, and GA_1 all increase levels gradually before sharply dropping off at the transition from vegetative development to reproductive growth. The levels of GA_3, and GA_4 remain low during vegetative development and increase dramatically at the same transition (Revilla et al., 2007). More research is needed for the exact function of these hormones, but it seems likely that the cytokinins or GA_1 are the light stimulated flower repressors and IAA is the flowering promoter hypothesized earlier. As daylengths shorten to the proper amount, IAA increases to be the highest among all the hormones overpowering the flowering repressors and allowing flowering to occur. As in the grape it is likely that the gibberellins and cytokinins may work together to form the

inflorescence axis and trigger flower differentiation. Again, there is presently not enough research to confidently confirm or deny these ideas.

Therefore, in order to devise a protocol in which flowering could be controlled in an *in vitro* setting all three of these factors must be accounted for. Hop must be vernalized naturally outdoors during winter, in a temperature controlled facility, or artificially via gibberellic acid which mimics the effect of cold. Next an optimum daylength must be determined for the specific hop genotype. Once determined, it may be possible to make an attempt at 'natural' flowering by placing the hop in a properly timed incubator. Another option would be to hormonally induce flowering by adding hormones such as gibberellic acid or IAA to plant media thus mimicking the natural hormones produced by the plant. If successful the introduced hormones will trigger the natural flowering process of hops.

Most importantly, if a protocol for successful induction of early flowering, or forced flowering, is developed it would drastically benefit research involving genetic engineering. Experiments involving genetic engineering in the hop seek to modify traits in the cones/glands such as the acids in brewing or xanthohumol for pharmaceuticals as explained earlier. Induced early flowering would allow for much faster assessment of the effects of research involving gene annotation and genetic trait improvement. One example already mentioned involving gene annotation was the agrobacterium-mediated attempt to amplify xanthohumol production in hops. Using present methods a single attempt takes 2 ½ years, if we can induce flowering this process could be shortened to months. An example we have seen of genetic trait improvement is the Apogee experiment where application of the chemical seems to have altered gene

expression inducing higher yield and an increase of brewing beneficial compounds in the hop cones. These experiments are also lengthy, with only one trial being capable during a year as hops flowering only once each growing season. Induced early flowering would again be immensely beneficial in that trails could potentially be implemented on a monthly basis, over a 10-fold increase in research able to be done in a single year.

Outlook

Based on the literature above I had planned and attempted the below experiment to test the effects of the hormones GA_3 and N-dimethyl succinamic acid (B9) on hop flowering in the summer of 2010. However, issues with contamination and improper handling of callus resulted in no data being collected on the effects of said hormones. A retrial was to be conducted in the Fall of 2010; however, a lack of hop tissue available to my laboratory resulted in too little time being available thus keeping me from executing the proposed plan. Therefore I offer it up to other researchers in the hopes that this project and others like it continue after my graduation. (See appendix).

Should these experiments prove fruitful and hop flowering is brought under human command it could prove immensely beneficial for the brewing and pharmaceutical industries. Breweries experimenting with Apogee or other methods at increasing hop yield or α- and β-acid levels could test the product every few months rather than once a year. Also those attempting to create transgenic hops that overproduce xanthohumol would be able to test for results in a few months rather than after two years of rigorous effort in caring for the hops throughout the transition from young sterile shoots to adult hops growing in the greenhouse or outdoors. New

improved yield would allow for the cheaper manufacture of beer while faster R&D on hop acids would create a multitude of new “flavors” stimulating the beer market with new potential for revenue. Finally, and possibly most importantly, forced flowering could take decades off xanthohumol research, speeding the approach of a new and exciting branch of anti-cancer medications.

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Appendix

Media Preparation

Sterile hops plants are used to induce calli on basal media consisting of 4.33 g/L Murashige and Skoog salts, 20 g/L Dextrose, 1 mL/L Gamborg's Vitamin Stock, and 0.8% Micropropagation Grade 1 Agar Powder (Schwekendiek et al, 2009). The media is made by mixing the salts, dextrose, and vitamin stock in double distilled water of approximately 75% the total volume being made. The vessel containing the mixture is then filled to 90% total volume and the pH of the solution is adjusted to 5.8. Finally the agar powder is added and the media autoclaved. After sterilization the media must be allowed to cool until it the beaker is capable of handling without protective equipment. Once cooled 4.54 µM TDZ can be added. Adding the TDZ before cooling could result in the denaturization of the hormone.

Callus Growth

Calli is then grown on media plates until they turn green (4-6 weeks after induction). Settings for the incubation of tissues are a 16hr light/ 8hr dark cycle (24^{o}C light, 16^{o}C dark). After green calli is established, four calli will be placed in a RITA™ vessel supplemented with one of the hormone combinations and each combination should be replicated 5 times. This technique will test a total of 120 callus pieces, 20 pieces per concentration. The reference literature reported concentration of 2.89µM GA_3 and 15.6 mM B9 to be effective in flower induction. The B9 concentration referenced was for a spray treatment and I believe that a much lower concentration will be necessary to achieve results via tissue culture. Therefore, the following hormone concentrations are tested:

- No hormone
- 1x GA_3 (2.89µM)
- 0.1x B9 (1.56mM)
- 0.001x B9 (15.62µM)
- 1x GA_3 +0.1x B9
- 1x GA_3 + 0.001x B9

The calli are exposed to the hormones for one week and then grown under hormone-free conditions. If no effects are noted after one week it may be pertinent to extend the hormone treatment to two weeks. At maximum the calli should be exposed to hormones no longer than one month as previous experiments showed that tissue treated with 1x GA_3 for greater than one month are reduced to meristem tissue and have no chance of flowering (Schwekendiek et al., 2009). It is recommended that the effect of the treatment be recorded by taking weekly pictures and written observations. After four weeks (or earlier if appropriate) morphological changes in appearance can be investigated microscopically. Significant results are defined as any change in physiology from normal vegetative state, with positive results being the formation of reproductive structures (flowers, flower buds, cones, etc.).

It may also be pertinent to split the above experiment into two separate experiments. In the first it could be beneficial to test the ability of gibberellic acid to vernalize hops callus tissue. The tissues should be obtained from stem pieces and grown into mature callus using the same procedures outlined above. Upon entering RITA™ vessels the calli will be separated into two groups. One group will be exposed to gibberellic acid infused media for one week, while the second group will be exposed for two weeks. Each group will also test gibberellic acid concentrations of 0.01X, 0.1X, 1X, and 10X. After the predetermined time period the calli are to

be switched to regular liquid media and grown into shoots. Once the shoots reach a height of at least five centimeters they can be tested for genetic changes indicating a successful transition to a vernalized state. Once/if an effective range is found, the experiment can be further limited to determine optimal levels and duration of gibberellic acid exposure.

Once a vernalization procedure is determined the second experiment can take place. Vernalized hops shoots should be transferred to sterile soil beakers for further growth. While in these beakers spray treatments of B9 at concentrations beginning at 0.001X up to and including the tested value from Thomas and Schwabe's (1969) experiment (1X or 15.6mM). Plants should be sprayed once a week until flowering occurs or up to two months. If hop flowering is induced the experiment should again be repeated with limited ranges of B9 in order to determine optimum levels.

It would also be pertinent to see if other compounds are capable and/or more effective at inducing flowering in hops. Some compounds to test are the auxin IAA, the cytokinin t-Z, Apogee, and the chemical compound 2-chloroethyltrimethylammonium chloride (CCC) which is reported to promote femaleness and hasten flowering in hops (Pharis & King, 1985; Thomas & Schwabe, 1969).